ORIGINAL ARTICLE

Expression Analysis of Apoptosis-related Markers TP53, BCL-2, BAX and c-MYC in Female Genital Tract Sarcomas

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Background: Most female genital tract sarcomas are highly malignant and fatal. Their aggressive growth pattern and poor response to chemotherapy are the major causes of death. Deregulation of the apoptosis pathway is related to tumorigenesis and chemodrug resistance. The purpose of this study was to investigate the expression status and relationship of the apoptosis-related markers TP53, BCL-2, BAX and c-MYC in this group of tumors. In addition, correlations of these markers with clinicopathologic findings and their prognostic significance were also examined.

Methods: Paraffin blocks of female genital tract sarcoma tissue from 54 patients were obtained after pathology review. Protein expression of TP53, BCL-2, BAX and c-MYC was examined using immunohistochemical staining with standard procedures. A semiquantitative method was used to assess the staining result where scoring 1–3 was negative and 4–9 was positive for expression. The mutual relationships between TP53, BCL-2, BAX and c-MYC were examined. Associations between expression of the apoptotic markers and tumor stage as well as outcome were also analyzed.

Results: We found that all 4 of the apoptosis-related markers were frequently expressed in female genital tract sarcomas. Of the 54 cases, 24 (44%) were positive for TP53, 23 (43%) for BCL-2, 25 (46%) for BAX, and 30 (56%) for c-MYC. A significant positive association was observed between BAX and c-MYC ($p < 0.001$). There was no significant difference for the expression status of the 4 markers in early and late stage tumors. In prognostic analysis, overexpression of TP53, late stage, and age were significant prognostic factors in both univariate and multivariate analyses.

Conclusion: Since changes in TP53, BCL-2, BAX and c-MYC frequently occur in female genital tract sarcomas, deregulation of apoptosis appears to be involved in the pathogenesis of this group of tumors. This mechanism may occur early in tumorigenesis and include the c-MYC/BAX apoptotic pathway or BCL-2. However, TP53 mutation may play a crucial role in this process, and clinically, it could be used as a prognostic indicator. [*J Chin Med Assoc* 2008;71(12):628–634]

Key Words: apoptosis, BAX, BCL-2, c-MYC, genital tract sarcoma, TP53

Introduction

Female genital tract sarcomas are rare malignant gynecologic tumors. They may arise from the mesenchymal tissues of the whole female genital tract, namely, from the vulva to the Fallopian tubes, although most mesenchymal tumors occur in the uterine corpus. Despite the relatively low incidence of these tumors, they are usually highly malignant with the exception of a few specific types such as low-grade endometrial stromal sarcoma and adenosarcoma. For example, carcinosarcomas and other uterine sarcomas account for fewer than 4% of all cancers of the uterine corpus. However, in a national database, they comprised 26% of deaths due to uterine corpus malignancies.[1] An aggressive growth pattern with early lymphatic or hematogenous dissemination is a typical clinical course. The other reason for the poor outcome of these tumors may be

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their poor response to chemotherapy. The overall survival rate for carcinosarcoma, high-grade leiomyosarcoma, and high-grade endometrial stromal sarcoma is poor, and death often occurs within 1 or 2 years of diagnosis.[2]

Because of the rare occurrence of female genital tract sarcoma, there have been few reports of pathogenetic studies of such tumors, especially with regard to molecular analysis. Frequent TP53 mutation with resultant overexpression of TP53 protein has been noted in uterine and ovarian sarcomas.[3] Reports of other apoptosis-related factors such as BCL-2, BAX and c-MYC, however, are rare, and most of them focused on uterine sarcoma.[4–6] Because deregulation of the apoptosis pathway is related to tumorigenesis and chemodrug resistance, studying the expression status and relationship of these factors in this group of tumors is of vital importance. It may aid in the understanding of the mechanism of pathogenesis and why these tumors are clinically so aggressive. In addition, understanding molecular abnormalities in neoplasm may help in the design of anticancer agents for targeted therapy that can reverse defective molecules.[7–9]

The purpose of this study was to examine the protein expression of the apoptosis-related markers BCL-2, BAX and c-MYC in female genital tract sarcomas. The relationships among them and with TP53 mutation were analyzed. In addition, the correlations of these markers with clinicopathologic findings and their prognostic significance were also examined.

Methods

Tissue specimens and review

Joint Institutional Review Board approval was obtained prior to conducting this investigation. Fifty-four patients with primary or recurrent sarcoma of the genital tract were recruited. All of these patients underwent surgical intervention at either Taichung or Taipei Veterans General Hospital between 1992 and 2006 (43 patients received operation in 2000 or later). Their medical records were reviewed and the survival time was followed to either death or December 31, 2006. The stage of disease was assigned based on the International Federation of Gynecology and Obstetrics (FIGO) staging system for carcinoma of the vagina, cervix, uterus, fallopian tube or ovary according to the origin of the sarcoma. All histopathology slides were reviewed by pathologists at these 2 hospitals to confirm the diagnosis, and tumor-rich paraffin blocks were selected for immunohistochemical (IHC) study.

IHC staining

Paraffin sections of 4 μm were dewaxed, rehydrated, and then heated with citrate buffer (pH 6.0). After being rinsed in phosphate-buffered saline (pH 7.6) and preincubated in serum blocking solution (10% goat serum), slides were reacted with the primary antibodies, i.e. anti-TP53, BCL-2, c-MYC mouse monoclonal antibodies (DAKO, Carpinteria, CA, USA; clones DO-7, 124 and 9E10) and anti-BAX rabbit polyclonal antibody (DAKO). The anti-TP53 antibody is a recombinant human wild-type TP53 protein, which labels wild-type and mutant-type TP53 protein when accumulated in human neoplasias.

The antibodies were used at dilutions of 1:300, 1:150, 1:800, and 1:400 for TP53, BCL-2, c-MYC, and BAX, respectively. Following incubation with the biotinylated secondary antibody (Histostain-Plus kit; Zymed, South San Francisco, CA, USA) and peroxidase-labeled streptavidin (Zymed LAB-SA), slides were rinsed again and were developed with the enzyme substrate diaminobenzidine. Tumor tissues known to be positive for the specific markers were used as positive controls: they were colon carcinoma for TP53, lymphoma for BCL-2, cervical carcinoma for c-MYC, and prostate carcinoma for BAX. Another tumor tissue treated with the same procedures but which was not incubated with primary antibody was used as negative control.

Assessment of immunoreactivity

The expressions of BCL-2, BAX and c-MYC were scored according to the extent and intensity of cytoplasmic immunoreactivity using a semiquantitative evaluation.[10] When the extent of immunoreactivity in the cytoplasm of tumor cells was < 15%, it was scored as 1 point; if 15–50% of tumor cells were positive, 2 points; and 3 points if > 50% of tumor cells were stained in the cytoplasm. The intensity of cytoplasmic staining was subjectively graded as weak (1 point), moderate (2 points) or intense (3 points). IHC evaluation and scoring were determined by 2 of the authors (FSL and YJJ). The product of the extent score multiplied by the intensity score was the final IHC score of BCL-2, BAX and c-MYC. The same calculation was used for TP53, but only nuclear staining was regarded as positive. Cases that had a final IHC score < 4 were considered to be negative and those with final IHC score ≥ 4 were considered to be positive.

Statistical analysis

All statistical analyses were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). The correlations between the expression of each apoptotic marker and pathologic type as well as disease progression were

examined by Yate's correction of contingency or Fisher's exact test. Because the case number was limited, disease status was grouped into early stage (FIGO stages I/II) and late stage (FIGO stages III/IV) in the analysis. The relationships among TP53, BCL-2, BAX and c-MYC were examined using Spearman's correlation analysis. For survival analysis, the operation dates of the patients were considered as the start time of the research. Data on survival were censored if the patient was still alive at the time of the last follow-up. The relation between the expression of each apoptotic marker and outcome was examined by log rank analysis. Survival curves and median follow-up time among survivors were estimated according to the Kaplan-Meier method, and p values were derived by the log-rank test. Hazard ratios for the risk of death were computed using a Cox proportional hazards model. Factors that showed a relationship with survival in the univariate analysis ($p<0.05$) were entered into a multivariate Cox model. A p value less than 0.05 was considered statistically significant.

Results

Patient characteristics

Among the 54 cases with genital tract sarcoma, 26 were leiomyosarcoma, 14 were carcinosarcoma, 6 were endometrial stromal sarcoma (ESS), 3 were sarcoma botryoides, 3 were undifferentiated sarcoma, and 2 were adenosarcoma. Forty-seven tumors primarily originated from the uterine corpus. They were: leiomyosarcoma, 26 cases; carcinosarcoma, 11 cases; ESS, 5 cases; undifferentiated sarcoma, 3 cases; and adenosarcoma, 2 cases. Two cases of carcinosarcoma and 1 case of ESS were of ovarian origin. The remaining 4 tumors arose from the cervix (sarcoma botryoides: 2 cases), fallopian tube (carcinosarcoma: 1 case), and vagina (sarcoma botryoides: 1 case). Thirty patients had stage I/II disease and 23 patients had stage III/IV disease at the time of surgery. The stage was unknown for 1 patient with uterine leiomyosarcoma who received initial surgery at another hospital. Median patient age was 56 years (range, 3–80 years). The demographic characteristics of the patients are summarized in Table 1. The median overall survival for the 54 patients was 44 months (95% CI, 19–69), and median follow-up time for the censored patients was 38 months (range, 7–185 months).

IHC results

The results of IHC staining are summarized in Table 2. Of the 54 cases, 24 (44%) were positive for TP53, 23 (43%) for BCL-2, 25 (46%) for BAX, and 30 (56%) for c-MYC. Examples of the IHC staining are shown in Figure 1.

The protein expression patterns of these apoptotic markers in the subgroups of leiomyosarcoma and

Table 1. Patients' demographic characteristics

Median age, yr (range)	56 (3–80)
Tumor stage, *n* (%)	
I	27 (50.0)
II	3 (5.6)
III	19 (35.2)
IV	4 (7.4)
Unknown	1 (1.9)
Tumor histology, *n* (%)	
Leiomyosarcoma	26 (48.1)
Carcinosarcoma	14 (25.9)
Endometrial stromal sarcoma	6 (11.1)
Sarcoma botryoides	3 (5.6)
Undifferentiated sarcoma	3 (5.6)
Adenosarcoma	2 (3.7)
Primary site, *n* (%)	
Uterus	47 (87.0)
Ovary	3 (5.6)
Cervix	2 (3.7)
Fallopian tube	1 (1.9)
Vagina	1 (1.9)

Table 2. Protein expression of apoptosis-related markers in female genital tract sarcomas*

IHC staining	Total ($n=54$)	Leiomyosarcoma ($n=26$)	Carcinosarcoma ($n=14$)	p
TP53 (+)†	24 (44.4)	13 (50.0)	9 (64.3)	0.594§
TP53 (–)‡	30 (55.6)	13 (50.0)	5 (35.7)	
BCL-2 (+)	23 (42.6)	12 (46.2)	5 (35.7)	0.763§
BCL-2 (–)	31 (57.4)	14 (53.8)	9 (64.3)	
BAX (+)	25 (46.3)	11 (42.3)	9 (64.3)	0.320§
BAX (–)	29 (53.7)	15 (57.7)	5 (35.7)	
c-MYC (+)	30 (55.6)	17 (65.4)	9 (64.3)	1.000‖
c-MYC (–)	24 (44.4)	9 (34.6)	5 (35.7)	

**Data presented as n (%); †IHC staining score ≥ 4; ‡IHC staining score < 4; §Yate's correction of contingency; ‖Fisher's exact test.*

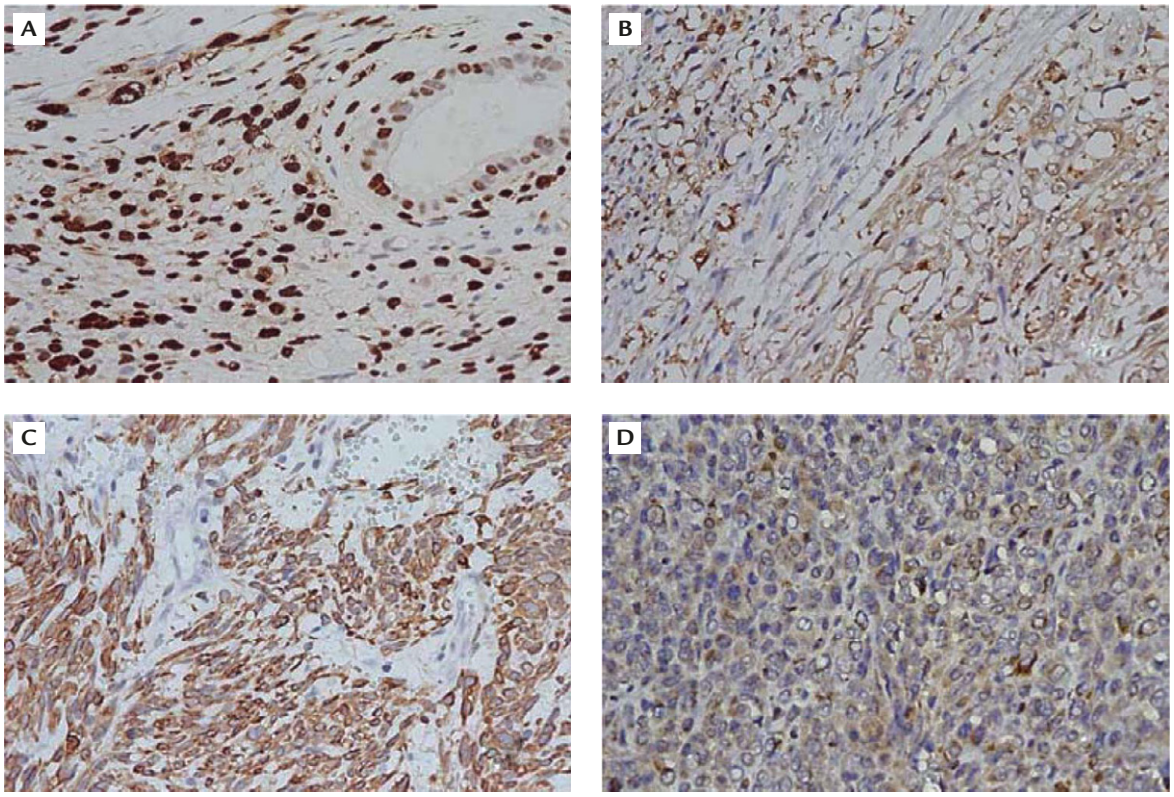

Figure 1. Immunohistochemical staining of apoptosis-related markers (400×): (A) TP53 in a carcinosarcoma (score: 9); (B) c-MYC in a carcinosarcoma (score: 9); (C) BCL-2 in a leiomyosarcoma (score: 9); (D) BAX in a leiomyosarcoma (score: 6).

Table 3. Spearman's correlation analysis of TP53, BCL-2, BAX and c-MYC expression

	TP53	BCL-2	BAX	c-MYC
TP53	1.000			
BCL-2	–0.081	1.000		
BAX	0.088	–0.226	1.000	
c-MYC	0.214	–0.141	0.497*	1.000

*$p < 0.001$.

carcinosarcoma are also shown in Table 2. No significant difference was noted between these 2 major types of sarcoma.

TP53, BCL-2, BAX, and c-MYC associations

The correlations among TP53, BCL-2, BAX and c-MYC were analyzed. BCL-2, BAX and c-MYC protein expressions were not associated with TP53 protein accumulation. There was also no correlation between BCL-2 and BAX, or between BCL-2 and c-MYC. However, a significant positive association was observed between BAX and c-MYC expression ($p < 0.001$) (Table 3).

Association of IHC staining and stage

To assess whether change in the apoptotic markers is associated with disease development, tumors were divided into early stage (stage I/II) and late stage (stage III/IV). Statistical analysis revealed no significant difference between expression status of the individual apoptotic markers and disease stage (Table 4).

Table 4. Correlation between stage and TP53, BCL-2, BAX and c-MYC expression*

IHC staining	Stage I/II ($n = 30$)	Stage III/IV ($n = 23$)	$p^{\dagger}$
TP53 (+)[‡]	15 (50.0)	9 (39.1)	0.610
TP53 (–)[§]	15 (50.0)	14 (60.9)	
BCL-2 (+)	13 (43.3)	10 (43.5)	1.000
BCL-2 (–)	17 (56.7)	13 (56.5)	
BAX (+)	13 (43.3)	11 (47.8)	0.962
BAX (–)	17 (56.7)	12 (52.2)	
c-MYC (+)	16 (53.3)	13 (56.5)	1.000
c-MYC (–)	14 (46.7)	10 (43.5)	

*Data presented as n (%); †Yate's correction of contingency; ‡IHC staining score ≥ 4; §IHC staining score < 4.

Overall survival analysis

Survival status information was available for each of the 54 patients, which included 29 deaths. Expression of TP53, BCL-2, BAX and c-MYC was compared in positive and negative groups for overall survival. Only patients with TP53-positive tumors had significantly

poorer survival than those with TP53-negative tumors ($p=0.039$) (Figure 2). In addition, patients with stage I/II had significantly longer survival than those with stage III/IV ($p=0.0016$) (Figure 3). In univariate analysis, age was another factor that correlated with survival, i.e. patients of older age had poorer prognosis ($p<0.05$ by Wald statistic). When TP53, stage and age were jointly analyzed using the Cox proportional hazards model with death as the endpoint, these 3 factors were still independent prognostic factors for overall survival (Table 5).

Discussion

It is widely known that cancer is a disease involving deregulated proliferation and programmed cell death. The net result of these 2 processes determines if the cancer cell number increases or decreases. Kinetic studies have implied that cell loss, rather than cell proliferation, indeed controls the rate of tumor growth.[11,12] For example, the fraction of cell loss in the range of 70% to more than 95% in most solid tumors has been reported.[13] Therefore, changes in the rate of cell death could have a major impact on tumor growth or regression.

The pathway of programmed cell death (also known as apoptosis) has been extensively studied and is delineated in review literature.[14–16] Briefly, the various signals that elicit apoptosis converge on the mitochondria, which in turn release cytochrome c into the cytosol, where it triggers activation of caspases, a series of enzymes considered to comprise the engine of apoptotic cell death, by formation of the adapter/caspase complexes, which then activate the downstream caspase cascade and finally cause cell death through selective destruction of subcellular structures, organelles, and the genome.

BCL-2 family proteins serve as critical regulators in the pathway of apoptosis. The apoptosis-suppressing BCL-2 gene promotes cell survival by blocking programmed cell death. It may do so by interfering

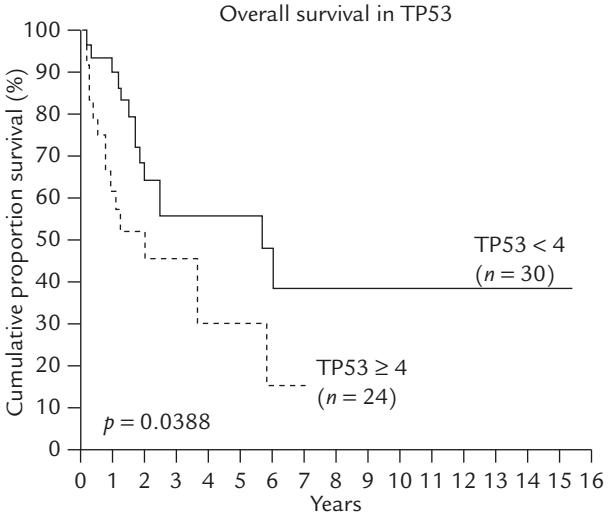

Figure 2. Kaplan-Meier survival curves of genital tract sarcoma stratified for TP53 expression status.

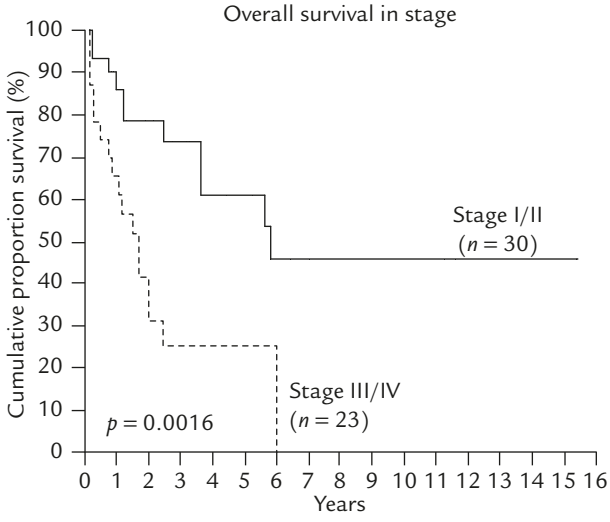

Figure 3. Kaplan-Meier survival curves of genital tract sarcoma stratified for tumor stage.

Table 5. Analysis of prognostic value of clinical factors and apoptotic markers

	Univariate Cox regression		Multivariate Cox regression	
	p*	HR (95% CI)	p	HR (95% CI)
Stage	0.003	3.27 (1.49, 7.14)	0.003	3.63 (1.56, 8.44)
Age	0.007	1.04 (1.01, 1.07)	0.019	1.04 (1.01, 1.07)
TP53	0.046	2.12 (1.01, 4.44)	0.004	3.37 (1.48, 7.69)
BCL-2	0.686	1.17 (0.56, 2.45)	NS	
BAX	0.147	1.73 (0.82, 3.65)	NS	
c-MYC	0.218	1.62 (0.75, 3.51)	NS	

**Wald statistic. HR = hazard ratio; CI = confidence interval; NS = not significant.*

with the mitochondria-mediated cell death pathway at 2 points, i.e. preventing cytochrome c release from mitochondria and interfering with the adapter protein Apaf-1.[17] To date, at least 15 BCL-2 family member proteins have been identified, including proteins that promote apoptosis such as BAX and BAD, and those that prevent apoptosis such as BCL-2 and BCL-XL.[18] These member proteins can act independently and they also have the ability to compete or inhibit one another in the apoptotic process. Overexpression of BCL-2 protein also prevents cell death induced by nearly all cytotoxic anticancer drugs and radiation, thus contributing to treatment failures in patients with some types of cancer.[19,20]

Tumor suppressor genes and oncogenes are also associated with the regulation and execution of apoptosis, most notably TP53 and c-MYC.[14–16] TP53 can promote apoptosis through the upregulation of BAX and the downregulation of BCL-2.[21] When TP53 is mutated, the functional inactivation of its protein product may result in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade. In addition to its well-documented growth-promoting property, c-MYC has been found to be a powerful inducer of apoptosis through TP53-dependent and independent pathways, and both pathways may facilitate cytochrome c release from mitochondria. On the other hand, BCL-2 allows c-MYC-induced proliferation to proceed without apoptosis and thus to potentiate its oncogenic action.

Although BCL-2 is involved in neoplastic transformation and conferred tumor resistance to chemotherapy, paradoxically, retrospective archival studies of non-small cell lung, breast, and ovarian carcinomas suggest that BCL-2 expression is associated with a survival advantage.[22–24] Similarly, in contrast to its apoptosis promoter function, high expression of bax in ovarian cancer has a negative impact on treatment response and survival.[25]

In this study, we examined protein expression of the apoptosis-related markers in female genital tract sarcomas. TP53, BCL-2, BAX and c-MYC were all frequently expressed in this group of tumors. This finding is compatible with previous observations in uterine sarcomas.[4–6] All of these protein changes occurred in both early- and late-stage diseases, with no difference in frequency. Therefore, changes in the apoptotic pathway and cell cycle deregulation may be early events in the development of this group of tumors. As far as the correlations among these markers are concerned, we noted a significant positive relationship between BAX and c-MYC. This finding is compatible with previous *in vitro* and *in vivo* studies that BAX is activated and required in c-MYC-induced apoptosis.[26–30] Albihn et al observed that etoposide- and doxorubicin-induced cell death was potentiated via c-MYC/BAX apoptosis.[31] The clinical significance of this finding remains to be investigated, however. In our 54 studied patients, 17 had tumors showing positive c-MYC and BAX. Only 3 patients are still alive with survival time from 18 to 135 months. In addition, their tumors were all in the early stage (1 cervical sarcoma botryoides, 2 carcinosarcomas). In another study, Taylor et al noted that oncogene-mediated induction of BAX was necessary but insufficient to enhance TP53-mediated apoptosis in rhabdomyosarcoma cells and that expression of all 3 proteins, MYC, BAX and TP53, was required for maximal cell death to occur.[32]

The prognostic significance of TP53, BCL-2, BAX and c-MYC was evaluated individually. Among these 4 markers, only expression of TP53 possessed a prognostic significance in both univariate and multivariate analyses, where patients with TP53-positive tumors had a poorer overall survival than those with TP53-negative tumors. This finding suggests that aggressive management in patients with TP53-positive tumors may be necessary even in the early stage.

In our study, BCL-2 was also expressed frequently in uterine leiomyosarcomas and other female genital tract sarcomas. However, it showed no association with the other 3 studied markers, nor did it have prognostic significance in these tumors. In the study of Leiser et al, BCL-2 positive leiomyosarcomas were associated with a longer time to recurrence in univariate analysis, but it was no longer an independent significant prognostic factor when evaluated in multivariate analysis.[6] Therefore, change in BCL-2 is likely as the c-MYC/BAX apoptotic pathway, although present, is not pivotal in the development of female genital tract sarcoma.

In conclusion, we observed that the apoptosis-related proteins TP53, BCL-2, BAX and c-MYC were overexpressed frequently in female genital tract sarcomas. Change in the expression of these proteins may occur early in the development of this group of tumors, in which the c-MYC/BAX pathway and BCL-2 may play roles in tumor apoptosis. However, TP53 mutation may be more pivotal in this process, and clinically, it could be used as a prognostic indicator.

Acknowledgments

This research was supported by a grant from Taichung Veterans General Hospital (TCVGH-956405C), Taiwan, R.O.C., and a private donation from the

family of Ms Ru-Chuan Ko Chang who succumbed to uterine carcinosarcoma.

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